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Virology 379 (2008) 306–313

Contents lists available at ScienceDirect

Virology

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Human T-cell leukemia virus type 1 Tax modulates interferon-α signal transduction through competitive usage of the coactivator CBP/p300

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ARTICLE INFO

Article history:
Received 2 May 2008
Returned to author for revision 18 June 2008
Accepted 30 June 2008
Available online 3 August 2008

Keywords:
HTLV-1
Tax
IFN-α signaling
CBP/p300 coactivator

ABSTRACT

We describe here Tax protein of human T-cell leukemia virus type 1 (HTLV-1) as an interferon (IFN)-α antagonist counteracting the transactivation function of IFN-stimulated gene factor 3 (ISGF3). Co-expression of Tax, but not the Tax mutant unable to bind to CBP, significantly inhibited the reporter gene expression directed by IFN-stimulated regulatory elements, despite that the formation of DNA-binding ISGF3 complex was unaffected. Gene activation induced by STAT2 transcription domain was also inhibited by expression of Tax. Furthermore, Tax-mediated transcriptional inhibition was reversed by overexpression of p300. These observations indicate that Tax interferes with IFN-α-induced JAK-STAT pathway by competition with STAT2 for CBP/p300 binding. Consistently, GST pull-down assay showed that Tax dose-dependently inhibited binding of STAT2 to p300. This study suggests that Tax may prevent IFN-α from exerting its antiviral, antiproliferative and proapoptotic effects, thereby contributing to persistent viral infection and HTLV-1-associated oncogenesis.

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus responsible for development of adult T-cell leukemia/lymphoma (ATLL) (Yoshida et al., 1984) and HTLV-1-associated neurodegenerative diseases such as myelopathy/tropical spastic paraparesis (HAM-TSP) (Gessain et al., 1985; Osame et al., 1986). ATLL is highly chemoresistant and its prognosis is usually poor. After conventional chemotherapy failure, interferon (IFN)-α combined with antiretroviral reagents such as zidovudine produces clinical remission followed by progression and death (Gill et al., 1995; Hermine et al., 1995). HTLV-1-associated oncogenesis has been demonstrated to be largely attributable to the expression of the viral regulatory protein Tax (Grassmann et al., 1989; Grassmann et al., 1992). In addition to its role in activating proviral transcription, Tax has been shown to activate transcription through protein–protein interactions with multiple transcription factors including the cyclic AMP-responsive element-binding factor (CREB), NF-κB, and the serum-responsive element-binding factor (SRF). Tax stimulates viral transcription through interacting with three conserved 21-bp repeat DNA elements known as viral CREs located within the HTLV-1 promoter. Tax binds to the CRE sequences in complex with the transcriptional factor CREB, which subsequently facilitates the transcription by recruitment of the cellular coactivators CBP (CREB-binding protein) and p300 (Giebler et al., 1997; Kwok et al., 1996).

CBP and p300 were originally identified as targets of CREB and adenovirus E1A, respectively (Chrivia et al., 1993; Eckner et al., 1994). They are large nuclear phosphoproteins that act as global transcriptional coactivators by binding numerous transcription factors (Janknecht and Hunter, 1996). CBP/p300 functions as coactivator or adapter by bridging transcription factors to general transcription machinery such as TFIIB, TBP and RNA polymerase II. In addition, CBP/p300 might contribute to transcriptional regulation by acetylation of chromatin via its intrinsic histone acetyltransferase (HAT) activity or its association with another HAT, p/CAF (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996). The simultaneous interaction of multiple transcription factors with CBP/p300 has been proposed to contribute to transcriptional synergy. Conversely, competition for limiting amounts of CBP/p300 has been suggested as a potential mechanism for transcriptional repression.

IFNs are a family of immunomodulatory cytokines that are produced in response to virus infection and possess both antiviral and antiproliferative functions. Secreted IFNs act in an autocrine or paracrine fashion to activate JAK-STAT pathway by binding to IFN receptors (IFN-α/β to IFNAR1/IFNAR2 and IFN-γ to IFNGR1/IFNGR2).

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doi:10.1016/j.virol.2008.06.035

The activated (phosphorylated) STAT proteins form homodimers, or heterodimers, with other STAT proteins, which subsequently translocate to nucleus and bind specific DNA sequences within the promoter region of IFN-stimulated genes (ISGs). In the case of signaling via IFN-α/β, heterodimer of phosphorylated STAT1 and STAT2 interacts with IFN regulatory factor 9 (IRF9; p48) to form the trimer transcription complex ISGF3 (IFN-stimulated gene factor 3). By recruiting the transcriptional coactivator CBP/p300, ISGF3 triggers the expression of a variety of genes driven by the promoter containing IFN stimulated response elements (ISRE). Viruses have evolved different strategies to subvert IFN response, mainly by interfering IFN induction, perturbing IFN signaling and inhibiting IFN-induced effectors. Viral proteins acting as IFN antagonists have been identified in a numbers of viruses belonging to different families. Additionally, employing its antiproliferative and proapoptotic properties, IFN has been used as a therapeutic agent for multiple hematological and non-hematological malignancies. The anti-tumor efficacy, however, is limited by emergence of IFN resistance, a phenomenon attributable to the functional defects involved in IFN signaling pathway (Sun et al., 1998).

Here we present evidence demonstrating that HTLV-1 Tax interferes with the JAK-STAT signal transduction pathway in response to IFN-α, and the mechanism underlying the antagonistic function involves interaction of Tax with coactivator CBP/p300 in competition with STAT2, thereby inhibiting the transcription activation of STAT2-containing ISGF3 complex.

Results

Inhibition of IFN-α-induced ISRE signaling by Tax

By analyzing the influence of Tax protein on replication of hepatitis C virus (HCV) replicon, we previously showed an attenuated IFN-α-induced antiviral response in Tax-expressing Huh-7 cells (Zhang et al., 2007). If Tax inhibits IFN-α response, interference could occur via global interruption of the JAK-STAT signal transduction pathway or via inhibition of specific ISG products such as protein kinase R or 2′,5′-oligoadenylate synthetase. To define a possible role of Tax in modulating IFN-α signaling, Tax- or Tax mutant-expressing plasmid was transfected into Huh-7 cells together with the reporter vector pISRE-luc to monitor ISRE-directed gene expression. Cells were either left untreated or treated with 100 IU/ml of human IFN-α 24 h later, and the luciferase activity was measured following a further incubation for 24 h. As shown in Fig. 1A, both the wild type and the Tax mutant m148, previously demonstrated to be functional in activating CREB pathway while failing to activate NF-κB pathway, significantly inhibited IFN-α induced ISRE-directed luciferase expression, while the Tax mutant m319, unable to activate CREB pathway although retaining the ability to activate NF-κB pathway (Yamaoka et al., 1996), had no significant effect on ISRE-driven gene expression, indicating that Tax inhibits ISRE-directed gene expression and its competence for signaling through CREB is important for the observed inhibition. Western blot analysis showed a comparable expression of Tax and Tax mutants (Fig. 1B). Similar results were also observed with Jurkat T cells (Fig. 1C), suggesting that Tax-mediated inhibition of JAK-STAT signaling in response to IFN-α was not cell-type specific. These results suggest that Tax interferes with IFN-α response by a global transcriptional repression of downstream effector gene expression.

Tax does not affect the formation of DNA-binding ISGF3 complex

As forementioned above, IFN-α signaling through the JAK-STAT pathway involves a series of events including tyrosine phosphorylation of STAT1 and STAT2, formation and nuclear translocation of ISGF3 followed by its binding to ISREs. Impairment at any of these steps might consequently lead to blockade of IFN-α-induced signaling. To understand the mechanism employed by Tax to counteract IFN's

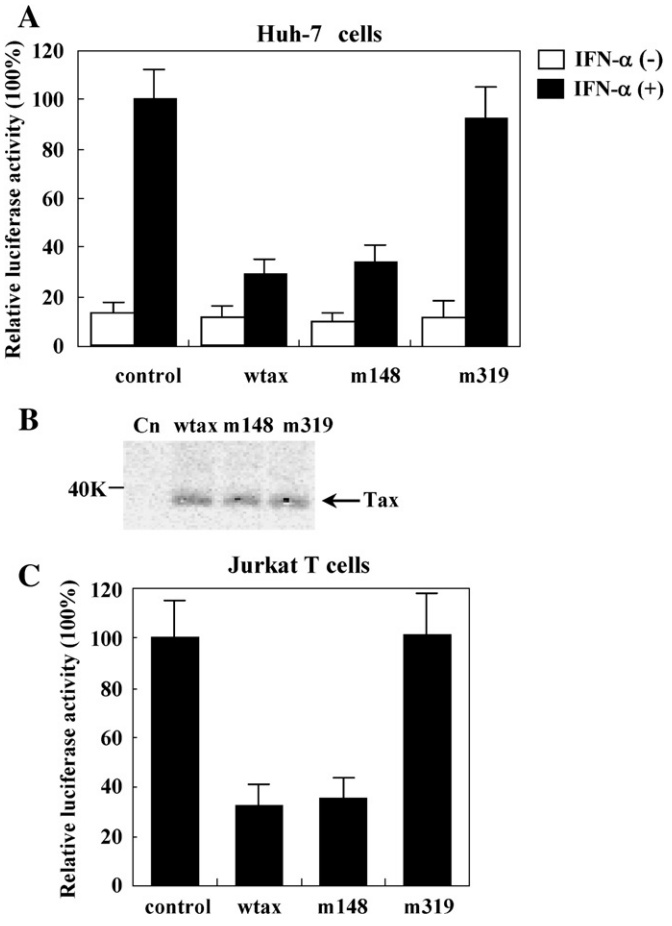

Fig. 1. Effect of Tax or its mutants on IFN-α-induced ISRE signaling. Huh-7 (A) or Jurkat T cells (C) were transfected with ISRE-luc together with pCn, pCnwtax, pCnm148, or pCnm319. Cells were either left untreated or treated with 100 IU/ml of human IFN-α 24 h later, and the luciferase activity was measured following a further incubation for 24 h. Renilla luciferase activities from cotransfected pRL-TK were used to normalize the transfection efficiency. Normalized luciferase activity from an otherwise identical control transfection with pCn backbone vector was set as 100%, and those in other transfectants are expressed as relative percentage. Results are presented as the means and standard deviations of four independent triplicate transfections. (B) Expression of Tax protein in each transfectant was confirmed by Western blot analysis. Solid arrow indicates the signals of Tax protein.

actions, we next investigated the formation of ISRE-binding ISGF3 in Huhwtax. Huhwtax is a cell line stably expressing Tax at a level comparable to HTLV-1-infected MT-2 cells (Fig. 2B), which was established by transfection with pCnwtax followed by G418 selection and limiting dilution (Zhang et al., 2007). Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay (EMSA) using the labeled ISRE as an oligonucleotide probe. As seen in Fig. 2A, in IFN-α-treated HuhCn cells, which were stably transfected with the empty vector pCn and served as a negative control, ISRE–protein complexes of delayed electrophoretic mobility was detected (lane 2), which was diminished by addition of antibody against STAT1 or STAT2 (lanes 7–8) but not by irrelevant antibody to β-actin (lane 9), confirming the identity of the ISRE-binding factor as ISGF3. The formation of ISRE–protein complex was significantly inhibited by addition an excess of unlabeled ISRE as a competitor (lane 3), revealing the specificity of the observed ISRE–protein binding. Unexpectedly, IFN-α-induced ISGF3 formation in Huhwtax was comparable to that detected in HuhCn (lanes 4–6). While expression of Tax conferred an attenuated IFN-α-induced antiviral response in Huhwtax cells (Zhang et al., 2007), the formation of ISRE-binding ISGF3 complex in their nuclear extract was not affected, which suggests that Tax inhibits IFN-

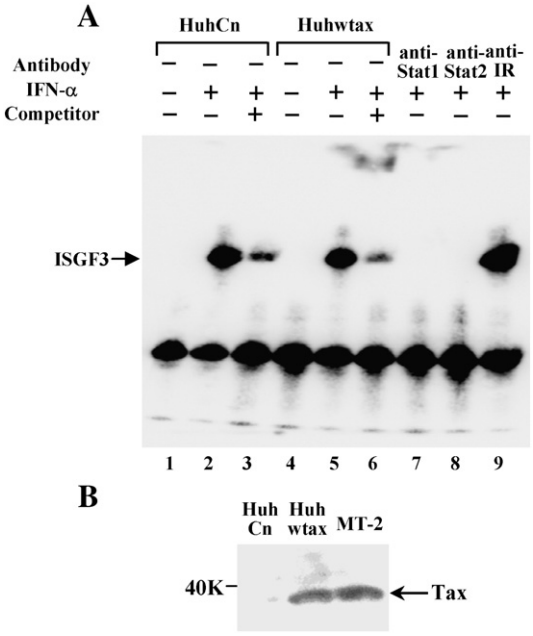

Fig. 2. Tax does not affect the formation of ISGF3 complex on ISRE promoter. (A) HuhCn or Huhwtax cells were treated in the absence or presence of 500 IU/ml of human IFN-α for 30 min. Nuclear extracts were prepared for EMSA analysis using labeled ISRE probe. In lanes 3 and 6, 50-fold molar excess of unlabeled ISRE competitor were included. In lanes 7–9, nuclear extracts were pre-incubated with the antibodies against STAT1, STAT2 or irrelevant β-actin (anti-IR) for 30 min prior to the addition of labeled probe. (B) Stable expression of Tax protein in Huhwtax cells was confirmed by Western blot analysis. Solid arrow indicates the signals of Tax protein.

α signaling without derailing the events involved in the formation of DNA-binding ISGF3 complex.

Tax represses transactivation function of STAT2

The results presented above imply that Tax averts IFN-α function via a mechanism other than inhibiting the activation and/or nuclear translocation of ISGF3 components. It is known that the C terminus of STAT2 serves as a transcriptional activation (TA) domain, which is essential for ISGF3 function. We next examined whether Tax could inhibit the transactivation function of STAT2. To this end, we constructed a vector pGAL4-Stat2(TA) by fusing the GAL4 DNA binding domain with the C-terminal 181 residues-coding region of STAT2. Expression of reporter gene from pGAL4-luc vector in HeLa cells was significantly induced by cotransfection with pGAL4-Stat2 (TA), but not by pGAL4-Stat2(TA)Δ with C-terminal 83 amino acids deleted, confirming the transactivation potential of the C terminus of STAT2 (Fig. 3). Importantly, cotransfection with Tax-expressing plasmid markedly inhibited the GAL4-Stat2(TA) transactivation activity, resulting in an approximately five- to six-fold lower luciferase expression when compared with the transfection in the absence of Tax. In contrast, the activation of the transcription from pGAL-luc by GAL4-VP16, which contains the activation domain of VP16 instead of Stat2 in GAL4-Stat2(TA), was not obviously affected by Tax protein, indicating that Tax-mediated inhibition of STAT2 transactivation is not due to an impairment of the general transcription machinery in Tax-expressing cells. Additionally, this result further excluded the possibility that Tax-mediated inhibition of ISGF3-directed transcription was attributable to the perturbation of events such as nuclear translocation because both GAL4-Stat2(TA) and GAL4-VP16 utilizes the same nuclear localization signal of GAL4. Taken together, these results suggest that Tax protein inhibits IFN-α signaling via repressing STAT2 transactivation.

Tax down-modulates IFN-α signaling by competitive binding to CBP/p300 coactivator

It has been demonstrated that STAT2 and STAT2-containing ISGF3 function in transcriptional activation partly through recruitment of CBP/p300 (Bhattacharya et al., 1996). Likewise, Tax also utilizes CBP/p300 for implementing its transcriptional activation competence, and moreover, Tax has been shown to inhibit CBP/p300-mediated transcription by competitive usage of the coactivator. Accordingly, a possible mechanism for Tax-mediated inhibition of IFN-α-activated ISGF3 transactivation is through binding to CBP/p300 in competition with STAT2. If it is true, the binding ability of Tax to CBP/p300 should be crucial for its repression of ISGF3 transactivation. To test this hypothesis, we investigated whether the mutant TaxK88A, defective in CBP/p300 binding, could exert similar repressive effect on ISGF3-directed transcription. As shown in Fig. 4A, the inhibitory effect of Tax on IFN-α-stimulated luciferase expression from pISRE-luc was completely abrogated by the mutation introduced in TaxK88A, revealing a correlation between CBP/p300-binding and ISGF3 repression. Similar result was obtained from the reporter assay with pGAL4-Stat2(TA) and pGAL4-luc, confirming that the binding of Tax to CBP/p300 is responsible for the inhibition of STAT2 and ISGF3 transactivation by Tax. Comparable expression of Tax and TaxK88A in each transfectant was confirmed by Western blot analysis (Fig. 4B).

To further verify the hypothesis that competition for limiting CBP/p300 is a mechanism for the observed Tax-mediated ISGF3 inhibition, we

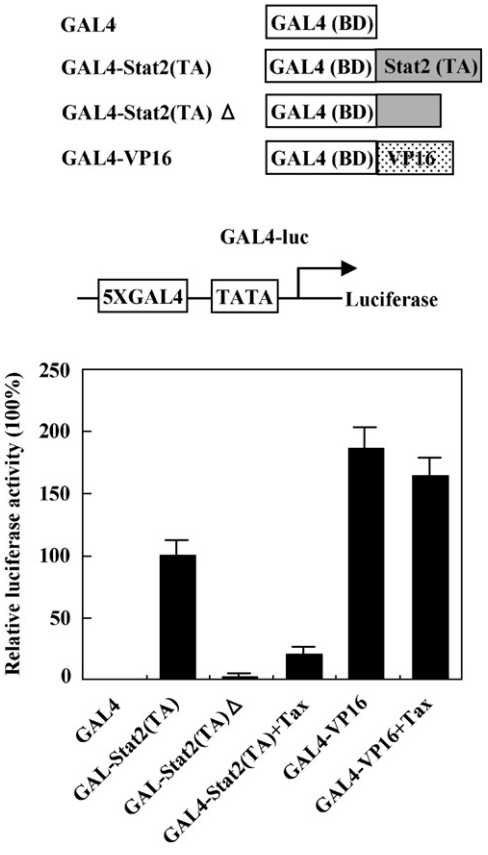

Fig. 3. Inhibition of STAT2 transactivation function by Tax. GAL4 carries the DNA binding domain only; GAL4-Stat2(TA) and GAL4-VP16 contain the transactivation domain of STAT2 and VP16 fused directly with the binding domain of GAL4; GAL4-Stat2(TA)Δ is a mutant of GAL4-Stat2(TA) with C-terminal 83 amino acids of STAT2 deleted. HeLa cells were transfected with the indicated expression constructs together with the GAL4-luc reporter vector, and luciferase activities were determined and calculated as described for Fig. 1. The results are from four independent triplicate experiments.

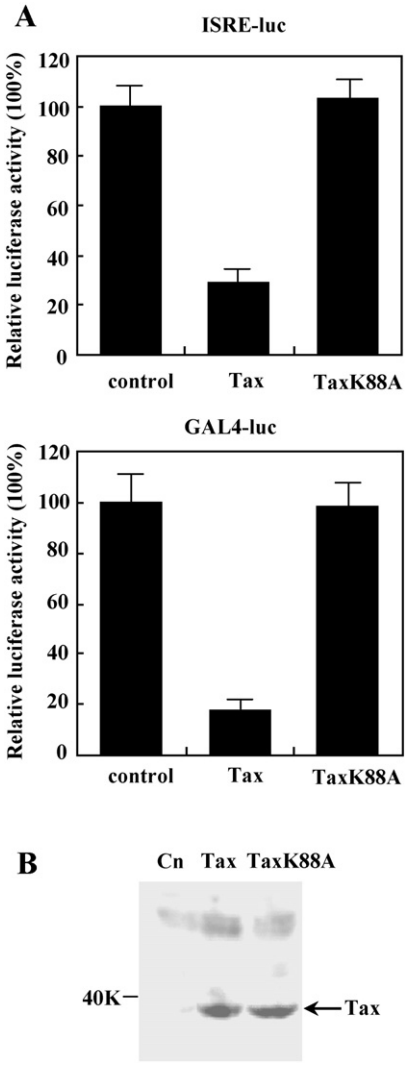

Fig. 4. Tax's ability to bind to CBP is critical for its repressive effect on STAT2 transactivation. (A) Huh-7 cells were transfected with ISRE-luc (upper) or GAL4-luc (lower) together with pCn, pCnwtax or pCnTaxK88A. Mutant TaxK88A harbors an amino acid substitution at condon 88 from lysine to alanine and is defective in CBP/p300 binding. Cells were treated with 100 IU/ml of human IFN-α 24 h later, and the luciferase activity was measured following a further incubation for 24 h. Renilla luciferase activities from cotransfected pRL-TK were used to normalize the transfection efficiency. Normalized luciferase activity from an otherwise identical control transfection with pCn vector was set as 100%, and those in other transfectants are expressed as relative percentage. Representative data are from five independent triplicate transfections. (B) Similar level of Tax expression in cells transfected with pCnwtax or pCnTaxK88A. Cells transfected with each indicated construct were harvested 48 h posttransfection, and Western blot analysis was performed using anti-Tax antibody (AS-5703). Solid arrow indicates the signals of Tax protein.

next investigated whether exogenously expressed CBP/p300 could restore the reduced ISGF3 transcriptional activity by Tax. In the absence of Tax, expression of p300 enhanced ISRE-directed luciferase gene expression in response to IFN-α (Fig. 5A), indicating a rate limiting level of p300 within the nucleus. Noteworthily, cotransfection of p300 expression plasmid completely restored the Tax-mediated inhibition of ISGF3 transactivation. Western blot analysis showed a similar Tax expression in Huh-7 cells irrespective of whether p300 was expressed or not (Fig. 5B), excluding the possibility that derepression of ISGF3 function by overexpression of p300 is due to reduced Tax expression in cells overexpressing p300. Together, these results strongly suggest that Tax-mediated inhibition of ISGF3 transactivation potential is mediated by competitive usage of CBP/p300 between Tax and STAT2.

Competition between Tax and STAT2 for p300 binding

To provide further evidence supporting the competition model proposed above, we next determined whether Tax could bind to p300 in competition with STAT2 in vitro. GST pull-down assay was performed using the GST-p300$^{302-661}$ fusion protein containing GST fused to amino acids 302–661 of p300, which encompasses the homologous region of the CH1 and KIX domains of CBP. Purified GST-p300$^{302-661}$ was bound to glutathione-agarose beads and then incubated with labeled in vitro translated Tax or STAT2 protein. Purified GST was included as a negative control. As expected, either Tax or STAT2 bound well to GST-p300$^{302-661}$ (Fig. 6, lanes 3–4), while no interaction was detected with GST alone (Fig. 6A, lanes 1–2). Notably, inclusion of increasing amount of purified Tax protein in the binding mixture dose-dependently reduced STAT2 binding to GST-p300$^{302-661}$ (Fig. 6A, lanes 5–7). This finding indicates that Tax competes with STAT2 for binding to p300. To exclude the possibility that Tax could interact directly with STAT2 and thereby prevent STAT2 from binding to p300, the binding mixture was subject to immunoprecipitation with anti-Tax antibody. STAT2 was not co-precipitated with anti-Tax antibody (Fig. 6B, upper), whereas association of p300 with Tax was detected under the same condition (Fig. 6B, lower), indicating that Tax did not directly interact with STAT2. Together, these data support a competition between Tax and STAT2 for p300 binding, which may account for the observed repression of STAT2 by Tax.

Tax-mediated inhibition of JAK-STAT signaling is specific for IFN-α

In addition to STAT2, STAT1 has also been shown to interact with CBP/p300, which plays a role in IFN-γ-induced signal transduction. To address whether Tax might affect IFN-γ signaling via competition with STAT1 for binding to CBP/p300, Tax-expressing plasmid was transfected into Jurkat T cells together with pGAS-luc, a reporter

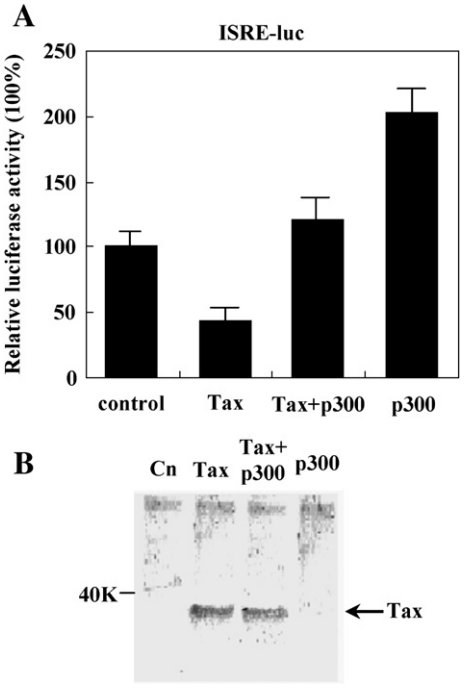

Fig. 5. Tax-mediated inhibition of STAT2 transactivation was reversed by overexpression of p300. (A) ISRE-luc reporter vector was transfected into Huh-7 cells together with pCn, pCnwtax and/or p300. Relative luciferase activities were determined and calculated as described for Fig. 1. Representative results are from three independent triplicate transfections. (B) Comparable Tax expression in cells irrespective of p300 co-expression was confirmed by Western blot analysis.

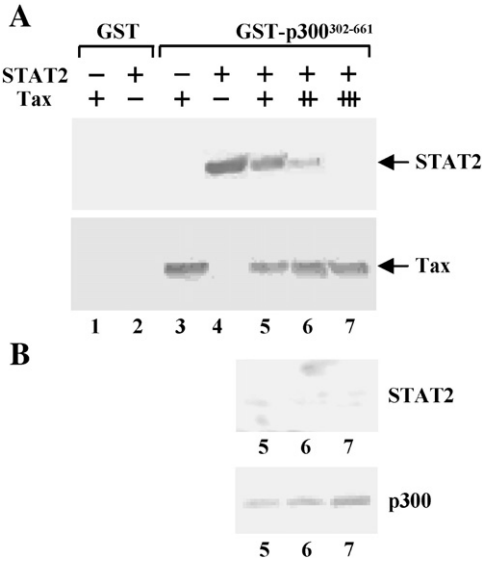

Fig. 6. (A) Tax inhibits STAT2 binding to p300. Labeled STAT2 or Tax in vitro translation product was incubated with GST alone (lanes 1–2) or GST-p300$^{302-661}$ (lanes 3–4). In competition assay (lanes 5–7), STAT2 was incubated with GST-p300$^{302-661}$ in the presence of increasing amount of Tax. (B) Tax does not directly interact with STAT2. The same reaction mixture was immunoprecipitated with anti-Tax antibody followed by Western blotting with anti-Stat2 (upper) or anti-p300 (lower) antibody.

vector encoding the luciferase gene under the control of tandem repeats of gamma-activated sequence (GAS) element that is recognized by STAT1 homodimer induced by IFN-γ. Cells were cultured in the absence or presence of 500 IU/ml of human IFN-γ 24 h later, and the luciferase activity was measured after an additional incubation for 24 h. As shown in Fig. 7A, the luciferase expression was significantly induced by treatment with IFN-γ, indicating the presence of functional IFN-γ receptor and STAT1 in Jurkat T cells. IFN-γ-induced luciferase expression was not significantly affected by cotransfection with either Tax- or TaxK88A-expressing plasmid. The luciferase expression under the control of HTLV-I LTR, which was monitored under the same condition, was substantially stimulated by cotransfection of Tax expression plasmid, suggesting that Tax was expressed and its function was intact in Jurkat T cells treated with IFN-γ (Fig. 7B). Further, expression of Tax was confirmed by immunoprecipitation and subsequent Western blot analysis with the antibody respectively recognizing the N and C terminus of Tax protein (data not shown). Together with the data shown in Fig. 1, it is suggested that Tax specifically modulates the JAK-STAT pathway in respond to IFN-α while having no effect on the signaling via IFN-γ.

Discussion

CBP/p300 regulates a variety of transcription factors involved in multiple signal transduction pathways, and competition between various classes of transcription factors for limiting amounts of CBP/p300 has been suggested as a repression mechanism to coordinate the transcriptional regulation of relevant genes. The transcriptional outcomes of simultaneous stimulation of two or more signal transduction pathways would depend on the abundance of the transcriptional factors and their relative affinities for CBP/p300. Inhibition of Ap1-dependent gene activation by steroid receptors and STAT1, for example, has been demonstrated to be attributable to competitive usage of CBP/p300 (Horvai et al., 1997; Kamei et al., 1996). Similarly, several oncogenic viruses encode viral proteins that bind to CBP/p300 and consequently deregulate CBP/p300-mediated transcription. HTLV-1-encoded Tax protein is among the best known of these. Indeed, multiple studies have linked Tax's binding to CBP/p300 with its repressive effect on the transcription directed by p53 (Ariumi et al., 2000), p73 (Ogryzko et al., 1996), c-Myb (Dai et al., 1996), and c-Jun (Van Orden et al., 1999), which also utilize CBP/p300 as the coactivator. Tax deregulation of the CBP/p300-mediated transcription, most of which are involved in apoptosis, cell cycle regulation, and differentiation, has significant implication in HTLV-1-associated malignant transformation.

Here, we investigated the effect of Tax protein on IFN-α-mediated signal pathway. Precedence for this study comes from our previous observation showing an attenuated IFN-α-induced antiviral response in Tax-expressing cells (Zhang et al., 2007), providing an explanation for the clinical study revealing a significantly lower rate of sustained IFN-α response in HCV/HTLV co-infected individuals, relative to those infected with HCV alone (Kishihara et al., 2001). The results presented here indicate that Tax interferes with IFN-α-induced JAK-STAT pathway, and the molecular basis for Tax-mediated IFN-α inhibition appears to be the competition between Tax and STAT2 for coactivator CBP/p300. We inferred this conclusion based on the following observations. Firstly, Tax's abilities to activate CREB-dependent pathway (Fig. 1A) and to bind CBP (Fig. 4A) are necessary for its repression on ISGF3-directed transcription. Secondly, in the presence of Tax, the formation of DNA-binding ISGF3 complex is unaffected (Fig. 2A) nevertheless being functionally compromised in directing ISRE-driven gene expression. Thirdly, Tax dose-dependently inhibits the binding of STAT2 to p300 in vitro (Fig. 6A), and there is no direct interaction between Tax and STAT2 in immunoprecipitation assay (Fig. 6B). Finally, Tax also inhibits the transcriptional activity of GAL4-Stat2(TA) containing the transactivation domain of STAT2 fused with GAL4 DNA-binding domain (Fig. 3), and Tax repression on ISGF3 was restored by

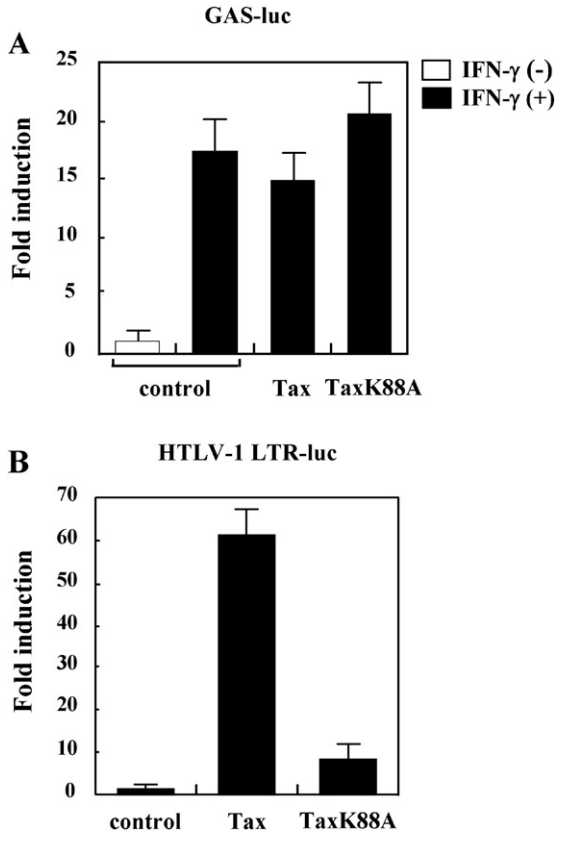

Fig. 7. Tax does not affect JAK–STAT pathway in response to IFN-γ signaling. Jurkat T cells were transfected with GAS-luc (A) or HTLV-1 LTR-luc (B) along with pCn, pCnwtax or pCnTaxK88A. Relative luciferase activities were determined as described for Fig. 1. Fold induction means the luciferase activity relative to that co-transfected with the control vector pCn. The results are from three independent triplicate experiments.

overexpression of p300 (Fig. 5A). Together, these findings strongly suggest that Tax competitively binds to CBP/p300 and disables its function in ISGF3-directed transcription.

Tax m319, which corresponds to the previously characterized M47 Tax mutant (Smith and Greene 1990), contains two amino acid mutations (L319R and L320S) in the carboxy-terminal domain. While Harrod et al. (1998) identified the KID-like domain around amino acid residues 81–95 as the major CBP-binding domain of Tax, Bex et al. (1998) reported that M47 mutant is impaired in CBP binding, thus explaining its inability to activate CREB pathway. A compromised interpretation is that the KID-like domain in Tax is critical for the recruitment of CBP/p300 but additional contact(s) might also be required for interaction of Tax with CBP/p300. Alternatively, the carboxy-terminal domain might play a role in maintaining the overall structure and/or promoting the conformational change to render the KID-like domain accessible to CBP/p300. If this is the case, the mutations in Tax m319 might attenuate the ability of Tax to sequester CBP/p300, thereby failing to suppress ISRE-luc expression. Actually, a similar observation was previously reported by Ariumi et al. (2000), who demonstrated that the mutations in Tax m319 abrogated the ability of Tax to repress p53-directed transcription by competitive binding to CBP. Further studies are now in progress to clarify this issue.

Repression of STAT2 by Tax is reminiscent of the scenario described in adenovirus-encoded E1A protein, which has been demonstrated to repress STAT2 transactivation and IFN-α-induced transcription at least in part through competition for CBP/p300 (Bhattacharya et al., 1996). In addition to these two viruses, viral proteins encoded by other oncogenic viruses, such as large T antigen from SV40 (Eckner et al., 1996) and polyomavirus (Cho et al., 2001), E6 protein from human papillomavirus (Patel et al., 1999), and Tat protein from HIV-1 (Ott et al., 1999), also bind to CBP/p300 and deregulate CBP/p300-mediated cellular transcription, it is thus probable, but remains to be proven, that an analogous anti-IFN strategy might also be employed by these viruses. It is known that interaction of Tax with the KIX domain of CBP/p300 is crucial for Tax-mediated coactivator recruitment, although additional contacts may further strengthen the interaction and contribute to Tax's transcription function. In the case of STAT2, previous studies have identified the CH1 domain as the binding site (Bhattacharya et al., 1996). CH1 domain is located immediately amino-terminal to the KIX domain. It is thus possible that steric hindrance or conformational changes following binding of Tax to CBP/p300 might result in the diminishment of STAT2 binding by blocking access of STAT2 to its binding sites. Additionally, it was reported that the transcriptional repression between Tax and p73β, c-Myb, c-Jun pathway is reciprocal (Lemasson and Nyborg, 2001; Van Orden et al., 1999). In a reporter assay to investigate whether STAT2 similarly repress Tax function, however, we found that overexpression of STAT2 didn't obviously affect Tax-stimulated HTLV-1 transcription (data not shown), suggesting that STAT2 could not inhibit binding of Tax to CBP/p300 and affect its ability to function as a coactivator in conjunction with CREB transcription factor. The failure of STAT2 to displace Tax from CBP/p300 is probably due to a higher affinity of Tax than STAT2 for CBP/p300. Alternatively, KIX domain may remain accessible for Tax binding and STAT2 displacement after binding of STAT2 to CH1 domain. Actually, a similar observation was previously reported by Riou et al., who demonstrated that Tax represses MyoD-dependent transcription by inhibiting MyoD-binding to the KIX domain of p300, but not vice versa (Riou et al., 2000). On the contrary, co-transfection of STAT2-expressing plasmid significantly inhibited Tax stimulation of HIV-1 transcription (data not shown), which involves activation of NF-κB pathway by Tax. Binding of STAT2 to CBP/p300 affects its ability to function as a coactivator in NF-κB- but not in CREB-dependent transcription, which may not be surprising considering that STAT2 binds to CH1 domain distinct from Tax and CREB, whereas both STAT2 and RelA of NF-κB share a common binding site (CH1) on CBP/p300. Consistent with this observation, binding of STAT2 to p300 in competition with RelA was demonstrated as the mechanism by which IFN-α inhibited TNF-α stimulation of HIV gene expression via NF-κB (Hottiger et al., 1998).

The data presented here indicate that Tax specifically deregulated the JAK-STAT pathway in respond to IFN-α but having no effect on the signaling via IFN-γ, suggesting that Tax did not interfere with the recruitment of CBP by STAT1. This is probably attributable to the fact that STAT1 interacts with the CH3 domain of CBP/p300 (Zhang et al., 1996), a region is relatively far from the binding site of Tax (KIX domain), and the CH3 domain may remain accessible for STAT1 binding after binding of Tax to KIX domain. Also, an alternative explanation for the different effects of Tax on IFN-α and IFN-γ signaling might be due to the difference in affinity of STAT2 and STAT1 for CBP/p300 binding, relative to that of Tax.

The ability of Tax to inhibit ISGF3 transactivation has potential biological implications. Like IFN-γ, IFN-α also plays a critical role in cancer immunosurveillance and immunoediting (Dunn et al., 2005). Impaired IFN-α signaling might therefore result in a loss of both the normal control of proliferation and regulation of apoptosis, thereby increasing the malignant behavior (Matin et al., 2001; Picaud et al., 2002). Indeed, reduced IFN-α-responsiveness has been linked to malignancy in various cancer types. For example, it was reported that deficiency in ISGF3 activity, due to suppressed expression of one or more components of ISGF3 complex, was involved in the pathogenesis of squamous cell carcinoma of the skin (Clifford et al., 2002). Further, it was demonstrated that stable expression of dominant negative STAT2 suppressed IFN-α-induced growth inhibitory response in highly IFN-α-sensitive human cells (Clifford et al., 2003). It is thus conceivable that repression of ISGF3 transactivation by Tax could contribute to the HTLV-1-associated malignancy by conferring a growth and/or survival advantage. Consistent with this hypothesis, it was previously reported that HTLV-1-infected T cells evade the antiproliferative action of IFN-β while having normal level of IFNAR1 expression and STAT phosphorylation (Smith et al., 1999). Additionally, ISGF3-directed transcription is a crucial step in transducing IFN-α-mediated antiviral response. The data presented here suggest that Tax-mediated ISGF3 inhibition might lead to a global repression of antiviral defense gene expression, thereby rendering unhindered viral replication. In addition to facilitating the replication of HTLV-1 itself, the antagonistic effect of Tax on IFN might also modulate the replication of other co-existing viral pathogens, providing a possible mechanism for the severer clinical consequence of viral infectious disease and poorer IFN-α responsiveness in patients co-infected with HTLV-1 (Boschi-Pinto et al., 2000; Kishihara et al., 2001). However, one concern in interpreting the physiological relevance of the results obtained here is that the level of Tax expression in HTLV-1-infected patients may be lower than that in established cell lines used here. In view that the titrating study was not performed here, it thus cannot be ruled out that Tax-mediated anti-IFN effect might be milder in vivo than that observed here.

While the manuscript was being prepared, Feng and Ratner reported that HTLV-1 down-regulates IFN-α-stimulated JAK-STAT signaling by reducing phosphorylation of tyrosine kinase 2 and STAT2, and provided evidence suggesting that Gag or Pr may be responsible for HTLV-1-mediated IFN-α inhibition (Feng and Ratner, 2008). Our data, however, do not support that Tax perturbs the events involved in the formation of DNA-binding ISGF3 complex. As shown in the result of EMSA (Fig. 2A), IFN-α-induced ISGF3 formation in Huhwtax was similar to that detected in HuhCn cells. Huhwtax constitutively expresses Tax protein at a level comparable to that in HTLV-1-infected MT-2 cells (Fig. 2B), and moreover this level of Tax expression conferred an attenuated IFN-α-induced antiviral response in Huhwtax cells (Zhang et al., 2007). Thus, the possibility that unaffected formation of ISRE-binding ISGF3 complex in Huhwtax was due to insufficient Tax expression in these cells was largely lessened, if could not be ruled out completely, allowing us to infer that Tax inhibits IFN-α signaling via a mechanism other than derailing the events involved in the formation of DNA-binding ISGF3 complex.

Nonetheless, the data presented here are not contradictory to the conclusion drawn from the Ratner's study, and it is possible that different HTLV gene products may act at different level of the JAK-STAT signaling to counteract IFN's action. Actually, it is frequently that viruses employ more than one strategy to evade IFN system.

In conclusion, we investigated here the effect of HTLV-1-encoded Tax protein on IFN-α signaling pathway, and provide evidence demonstrating that Tax negatively modulates IFN-α-induced JAK-STAT pathway by competing with STAT2 for coactivator CBP/p300. Tax preventing IFN-α from inducing an antiviral state and programming cell death provides an additional mechanism for the role of Tax in persistent viral infection and development of ATLL in HTLV-1-infected individuals, highlighting the significance of the anti-HTLV strategies targeting Tax protein.

Materials and methods

Plasmids

Empty plasmid pCn, and plasmids encoding wild-type HTLV Tax (pCnwtax) or its mutants (pCnm148 and pCnm319, K88A) have all been described previously (Yamaoka et al; 1996; Zhang et al., 2007; Harrod et al., 1998). The reporter vectors, pISRE-luc and pGAS-luc, were purchased from Stratagene. For construction of pGAL4-Stat2(TA), the cDNA of STAT2 transactivation domain (amino acids 670–851) was amplified with primers 5′-atctgcgatcgccatgCGGGATGAAGCTTTT-GGGTG-3′ and 5′-acctgtttaaacCTAGAAGTCAGAAGGCAT-3′, digested with Sgf I and Pme I, and ligated into in pFN11A(BIND) Flexi vector (Promega), which had been cut with the same enzymes immediate downstream of GAL4 DNA-binding domain. The cDNA insert for pGEX-p300 (amino acids 302–661) was generated by PCR using primers 5′-ataggatccatgGGTCAACAGCCAGCCCCG-3′ and 5′-agagaattctcaAG-CATTTGGTAGCATGTTCTG-3′, digested with BamH I and EcoR I and ligated into BamH I/EcoR I-digested pGEX-2Z (Amersham). The sequences of these constructs were confirmed by nucleotide sequencing.

Cells

Human hepatoma cell line Huh-7 and human cervical carcinoma HeLa cells were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 U/ml penicillin and streptomycin. Jurkat T-lymphocytes were maintained in RPMI1640 medium supplement with 15% fetal calf serum. The cell line Huhwtax, which stably expresses Tax at a level comparable to HTLV-1-infected MT-2 cells, was established by transfecting Huh-7 cells with pCnwtax followed by G418 selection and limiting dilution.

Transient transfection and luciferase assay

Cells were seeded at 1×10^5 in 1 ml medium per well of 12-well plates 24 h before transfection. Indicated plasmid DNAs were transfected into cells with FuGENE6 (Roche). For each transfection, pRL-TK (Promega) vector was cotransfected as an internal control to normalize the transfection efficiency. The cells were harvested at 48 h posttransfection, and the cell lysates were prepared for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Luciferase activities were measured using a TD-20/20 Luminometer (Promega).

EMSA

The probe used in the EMSA was obtained by annealing biotinylated consensus ISRE oligonucleotides $(AGGAAATAGAAACTG)_2$ and its complement. Nuclear extracts were prepared from the cells treated with or without IFN-α using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce). EMSA was performed with the Lightshift Chemiluminescent EMSA Kit (Pierce) and the signals were detected with streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate. For competition experiments, unlabeled double-strand oligonucleotides were used in a 50-fold molar excess. For supershift experiments, nuclear extracts were pre-incubated for 30 min with specific antibodies before addition to the binding reaction.

GST pull-down assay

GST-$p300^{302-661}$ fusion protein was induced by isopropyl-thiogalactopyranoside in BL21 *Escherichia coli* transformed with pGEX-p300, purified by binding to glutathione-Sepharose 4B (Bulk GST Purification Module, GE Healthcare), and dialyzed with PBS overnight. Tax and STAT2 proteins were in vitro translated with the TNT-coupled reticulocyte lysate systems kit (Promega) and labeled with Transcend tRNA (Promega). GST or GST-$p300^{302-661}$ was incubated with Tax or STAT2 in binding buffer. GS4B beads were added and incubated for 1 h. Proteins that bound to the beads were separated by SDS/PAGE and detected with streptavidin-horseradish peroxidase and chemiluminescent substrate.

Western blot analysis

Separation of protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed standard methods. After the proteins were transferred to Hybond-P PVDV Membrane (GE Healthcare), the membranes were blocked and then probed with monoclonal antibody specific for HTLV-I Tax (AS-5703, Microbix Biosystems Inc.), and signals were visualized with ECL Plus Western Blotting Detection Reagents (GE Healthcare).

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